Title: Enhanced T cell immunity to osteosarcoma via antibody blockade of PD-1/PD-L1 interactions

Running Title: PD-L1 blockade enhances immunity to osteosarcoma

Author names and affiliations: Danielle M. Lussier[1,2], Lauren O'Neill[4], Lizbeth M. Nieves[2,3], Megan S. McAfee[1,2], Susan A. Holechek[2], Andrea W. Collins[5], Paul Dickman[6,7], Jeffrey Jacobsen[6], Pooja Hingorani[5], and Joseph N. Blattman[1,2]

[1]Molecular and Cellular Biology Graduate Program, [2]Center for Infectious Diseases and Vaccinology, [3]Molecular Biosciences and Biotechnology, [4]Postbaccalaureate Research Education Program, School of Life Sciences, Arizona State University, Tempe, AZ, 85287
[5]Center for Cancer and Blood Disorders, [6]Department of Pathology and Laboratory Medicine, Phoenix Children's Hospital, Phoenix, AZ, 85016
[7]Department of Child Health, University of Arizona College of Medicine-Phoenix, Phoenix, AZ

Email contacts: Danielle M. Lussier (dmlussie@asu.edu), Lauren O'Neill (laoneill@asu.edu), Lizbeth M. Nieves (lmnieves@asu.edu), Megan S. McAfee (msmcafee@asu.edu), Susan A. Holechek (sholeche@asu.edu), Andrea W. Collins (awhite@phoenixchildrens.com), Paul Dickman (pdickman@phoenixchildrens.com), Jeffrey Jocobsen (jjacobsen@phoenixchildrens.com, Pooja Hingorani (phingorani@phoenixchildrens.com), Joseph N. Blattman (Joseph.Blattman@asu.edu)

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Corresponding Author: Address correspondence and reprint requests to Dr. Joseph Blattman, Arizona State University, BioDesign Institute, 727 E. Tyler St., Tempe, AZ, 85287. (480) 965-2909. Email address: Joseph.Blattman@asu.edu

1. Abstract:

Osteosarcoma is the most common bone cancer in children and adolescents. While 70% of patients with localized disease are cured with chemotherapy and surgical resection, patients with metastatic osteosarcoma are typically refractory to treatment. Numerous lines of evidence suggest that cytotoxic T-lymphocytes (CTL) limit the development of metastatic osteosarcoma. We have investigated the role of PD-1, an inhibitory TNFR family protein expressed on CTL, in limiting the efficacy of immune mediated control of metastatic osteosarcoma. We show that human metastatic, but not primary, osteosarcoma tumors express a ligand for PD-1 (PD-L1) and that tumor infiltrating CTL express PD-1, suggesting this pathway may limit CTL control of metastatic osteosarcoma in patients. PD-L1 is also expressed on the K7M2 osteosarcoma tumor cell line that establishes metastases in mice, and PD-1 is expressed on tumor infiltrating CTL during disease progression. Blockade of PD-1/PD-L1 interactions dramatically improves the function of osteosarcoma-reactive CTL *in vitro* and *in vivo*, and results in decreased tumor burden and increased survival in the K7M2 mouse model of metastatic osteosarcoma. Our results suggest that blockade of PD-1/PD-L1 interactions in patients with metastatic osteosarcoma should be pursued as a therapeutic strategy.

2. Introduction:

Osteosarcoma remains the most common pediatric bone cancer, and is the eighth most common childhood malignancy overall [1, 2]. Osteosarcoma develops from bone osteoblasts, typically during periods of rapid bone growth, with a median occurrence at 14 years of age [3]. Primary tumors typically occur in long tubular bones, with a small percentage of primary tumors originating in the axial skeleton [4]. Chemotherapy, often accompanied by surgical resection, can improve the outcome for patients with localized tumors, with a 5-year event-free survival (EFS) for treated patients of 65-70% [5-7].

Unfortunately, 25-30% of osteosarcoma patients present with metastatic disease at diagnosis and patients with non-metastatic osteosarcoma at initial presentation often develop metastatic disease [8, 9]. Osteosarcoma metastases most often occur in the lungs followed by other bones. Chemotherapy, with or without surgical resection, is not effective against metastatic osteosarcoma with a 5-year EFS for these patients of less than 20% [5-7]. Therefore, new efficacious treatment modalities for metastatic osteosarcoma are needed to improve patient prognoses.

T cells have the potential to potently and specifically reject cancerous cells while avoiding the unwanted side effects seen in other tumor therapy strategies. In many settings cancer patients generate T cell responses against their respective tumors, and tumor-reactive T cells are able to infiltrate the tumor to slow progression or eliminate the tumor [10, 11]. However, during tumor equilibrium or progression, tumor-reactive T cells often become tolerized, limiting their ability to reject tumors. This tolerance, often termed exhaustion, is characterized by a progressive decrease in T cell proliferation, cytokine production, and cytotoxic function [12].

T cell exhaustion was first shown in the lymphocytic choriomeningitis virus (LCMV) mouse model of chronic viral infection, and has since been confirmed in numerous clinical and experimental tumor settings including hepatocellular carcinoma, ovarian cancer, Hodgkin lymphoma, urothelial cell carcinoma, pancreatic cancer, renal cell carcinoma, malignant melanoma, acute myeloid leukemia, head and neck squamous cell carcinoma, and Friend leukemia virus induced tumors [13-20]. Thus, many next-generation immunotherapeutic approaches for targeting chemotherapy- and radiation-resistant tumors are aimed at re-invigorating T cell responses to mediate potent and specific tumor rejection.

Numerous lines of evidence suggest that tumor-reactive CTL are induced during the development of metastatic osteosarcoma but become exhausted: 1) Large numbers of CTL infiltrate metastatic osteosarcomas but are unable to mediate tumor rejection [21, 22], 2) polymorphisms associated with increased expression of CTLA4, a potent T cell inhibitory protein, are associated with higher risk of developing osteosarcoma [4], 3) metastatic tumors, but not primary osteosarcoma, have increased expression of ligands for T cell Ig/mucin molecule 3 (TIM3), which has been shown in other tumor settings to inhibit the function of infiltrating CTL, leading to tumor progression [23, 24], and 4) B7-H3 expression, a co-stimulatory protein involved in tumor immune escape from T cells, inversely correlates with CTL infiltration in human osteosarcoma, and is indicative of poor prognosis in osteosarcoma patients [25-28].

T cell exhaustion has been shown to be due, at least in part, to expression of inhibitory proteins on tumor-reactive T cells that are engaged by their respective ligands on tumor cells[29, 30]. Programmed death receptor-1 (PD-1, CD279) expression on

tumor-specific CTL, which binds PD-1 ligand (PD-L1, CD274) on tumor cells, has been shown to inhibit T cell function leading to tumor progression in a variety of experimental and clinical tumor settings, including hepatocellular carcinoma, ovarian cancer, Hodgkin lymphoma, urothelial cell carcinoma, pancreatic cancer, renal cell carcinoma, malignant melanoma, acute myeloid leukemia, and head and neck squamous cell carcinoma[14-20]. Antibody blockade of PD-1/PD-L1 interactions in experimental tumor settings can effectively restore CTL function and enhance immune-mediated rejection of many of these tumors [19]. Recently, a phase I/II clinical trial evaluation of the efficacy of PD-1 blockade in adult advanced cancers demonstrated minimal treatment side-effects with 17% of patients experiencing durable tumor regression and 41% with prolonged stabilization of disease [31]. However, no studies have evaluated the efficacy of PD-1/PD-L1 blockade in metastatic osteosarcoma. Therefore, we analyzed PD-1 expression on tumor-reactive T cells, PD-L1 expression on metastatic osteosarcoma, and the effects of blockade of PD-L1 on tumor-reactive T cell function in slowing metastatic disease progression.

We find that PD-L1 is expressed on human metastatic osteosarcoma tissue, but is not expressed on primary tumors from the same patients. Similarly, CTL infiltrating human metastatic osteosarcomas are positive for PD-1 expression, but no PD-1-positive CTL are observed in primary osteosarcomas. Using the K7M2 ectopic metastatic osteosarcoma mouse model, we show that these cells are similarly positive for PD-L1 and that after implantation and tumor generation, infiltrating CTL are PD-1 positive. Furthermore, we show that PD-1/PD-L1 interactions impair CTL cytokine production, while PD-L1 shRNA knock-down or antibody blockade improves T cell function *in vitro*.

In vivo antibody blockade of PD-L1 results in longer host survival and fewer pulmonary metastases during disease progression. Thus, given the success of this strategy in other tumor systems, blockade of PD-1/PD-L1 interactions should be pursued as an immunotherapy for pediatric patients with metastatic osteosarcoma.

3. Methods:

3.1. Antibodies and cell lines: Fluorochrome-conjugated anti-mouse monoclonal antibodies (Abs) specific for CD8α, CD274, CD279, tumor necrosis factor (TNF), interferon gamma (IFNγ), and interleukin-2 (IL-2) were purchased from eBiosciences (San Diego, CA). Anti-PD-1 and anti-PD-L1 monoclonal antibodies used for immunofluorescence were purchased from Abcam (Cambridge, MA). For depletion studies, the 2.43 hybridoma was purchased from ATCC (Manassas, VA). The anti-PD-L1 monoclonal antibody (clone 10F.9G2) used for *in vivo* blockade experiments was purchased from BioXCell (West Lebanon, NH). K7M2 osteosarcoma cells were purchased from ATCC.

3.2. Mice and generation of tumors: 3-4 week-old Balb/cJ mice were purchased from the Jackson Laboratories (Bar Harbor, ME) and maintained under specific pathogen-free conditions in Arizona State University Biodesign Institute animal facilities. All experiments were approved by the ASU Institutional Animal Care and Use Committee, and conducted under appropriate supervision. To establish metastatic osteosarcoma tumors in mice, 10^6 K7M2 cells were injected via the lateral tail vein in 100uL of Hanks

Balanced Salt Solution. Both weight loss and a clinical scoring system were used to monitor for the development of metastatic lung disease, with a mean time to diagnosis of 24 days from injection of cells. Mice were ranked from 0 (normal) to 3 (abnormal) in mentation/appearance, respiration, ambulation, and for the occurrence of tremors/convulsions. Mice were euthanized for analysis by CO_2 asphyxiation when weight loss was >10% and physical symptoms (a cumulative score>6 or a score of 3 in any individual category) were observed.

3.3. CD8 depletion *in vivo*: Supernatant from 2.43 hybridoma cells was precipitated in saturated ammonium sulfate to 45% (v/v) overnight at 4°C and dialyzed against PBS for 24 hrs. The concentration of dialyzed antibody was determined by UV spectroscopy, and 0.3 mg of purified antibody was administered via intraperitoneal injection twice before tumor inoculation (day -5 and -3), and continued every three days after inoculation until euthanasia. CD8 T cell depletion was confirmed by flow cytometry analysis of peripheral blood mononuclear cells, as previously described[32].

3.4. Metastatic lung preparation: Mice with metastatic pulmonary disease were anesthetized with 2μL/g of a dose of 42mg/kg ketamine, 4.8 mg/kg xylazine, and 0.6 mg/kg acepromazine followed by lung perfusion with ice-cold PBS to remove PBMC, mice were then euthanized. Lungs were collected in RPMI media and digested with collagenase. Osteosarcoma-infiltrating cells were isolated from lung tissue by centrifugation over a 30/90% Percoll gradient (Sigma-Aldrich, St. Louis, MO) and collection of interface cells before antibody staining and analysis of cell populations on

an LSRFortessa flow cytometer (BD Biosciences, San Jose, CA) [33]. Flow cytometry data were analyzed with FlowJo8.8 (Tree Star Inc., Ashalnd, OR) and graphs generated with Prism5 software (GraphPad Software, La Jolla, CA).

3.5. Intracellular cytokine staining: Lymphocytes were cultured alone or stimulated with K7M2 cells 10^6 cells/well. As a positive control for T cell function, lymphocytes were stimulated with 4T1 cells antigen-presenting cells (ATCC) pulsed for 4 hours with K7M2 tumor lysate. K7M2 tumor lysate was prepared by 5 freeze/thaw cycles with dry ice and ethanol, followed by centrifugation at 1900 RPM for 10min at 4°. GolgiStop (BD Biosciences) was added at 1 hour to inhibit export of cytokines and after a further 5 hours of incubation, cells were stained for extracellular proteins [34]. Permeabilization and intracellular staining for cytokines was done according to manufacturer's instructions using the Cytofix/Cytoperm kit (BD/Pharmingen).

3.6. Cytotoxicity ELISA: Lymphocytes were isolated from lung tissue, and cultured alone, with K7M2 cells at varying effector to target cell ratios, and with antigen presenting cells (4T1 cells) non-pulsed or pulsed at varying concentrations. LDH Elisa was performed using CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI) and absorbance was recorded at 490nm.

3.7. *In vivo* PD-L1 antibody blockade: Mice inoculated with K7M2 cells as described above were additionally administered 200ug PD-L1 antibody (10F.9G2) in PBS or PBS

control intraperitoneally every three days, for a total of 5 injections, starting one day after tumor inoculation [35].

3.8. Immunofluorescence of human osteosarcoma: Formalin-fixed paraffin-embedded matched primary and metastatic osteosarcoma tumors from patients were obtained from the Phoenix Children's Hospital after IRB approval. 5μm thick tissue cryostat sections were deparaffinized and hydrated using xylene and ethanol dilutions, then incubated for 30 min at 85°C in 1x citrate buffer followed by blocking in Image IT (Life Technologies) reagent for 30 min at 22°C. Primary antibodies against PD-1 or PD-L1 (1:200 dilution) were incubated on slides overnight at 4°C in a moist chamber, followed by incubation with anti-mouse secondary antibody (1:750 dilution) conjugated to Texas Red or Alexa Fluor 488 fluorochromes, respectively. Sections were counterstained with DAPI (1:2000 dilution) and mounted. A Leica TCS SP5 confocal microscope was used to visualize tissue staining and produce images. Images are shown at maximum intensity projections of all visualization planes within 1μm confocal sections.

3.9. Immunofluorescence of mouse osteosarcoma: After lung perfusion, tissue was fixed in 10% formalin, followed by 30% sucrose for cryoprotection. Tissue was then rapidly frozen using OCT Tissue Tek (Fisher Scientific), cut into 10 micron sections, and mounted on charged microscope slides (Fisher Scientific). Tissue was then incubated for 30 min at 85°C in 1x citrate buffer followed by blocking in Image IT (Life Technologies) reagent for 30 min at 22°C. Primary antibody against PD-1 (1:750 dilution) was incubated on slides overnight at 4°C in a moist chamber, followed by incubation with

anti-mouse secondary antibody (1:1000 dilution) conjugated to Texas Red. Sections were counterstained with DAPI (1:2000 dilution) and mounted. Tissues were visualized as described above.

3.10. PD-L1 knock-down in K7M2 cells: 293FT cells were purchased from ATCC, and were plated at a low confluency on 10cm dishes. The ViraPower Lentiviral Expression System, purchased from Life Technologies (Carlsbad, CA), was incubated with the CD274-set siRNA/shRNA/RNAi Lentivector, purchased from Applied Biological Materials (Richmond, BC), to transfect 293FT cells and incubated overnight at 37° prior to harvesting the virus. Virus was used to transduce K7M2 cells by incubating for 48hrs at 37°. Once the transduced K7M2 cells were confluent, cells were stained with anti-PD-L1, and sorted for PD-L1 negative cells using a FACSAria flow cytometer (BD Biosciences).

4. Results:

4.1. Metastatic, but not primary, human osteosarcoma tumors express PD-L1.

In order to determine if PD-L1 blockade should be pursued as a treatment for metastatic osteosarcoma patients, we first asked whether PD-L1 is expressed on human metastatic tumors. Matched primary and metastatic osteosarcoma samples from eleven pediatric patients (Table 1) were obtained and stained for PD-L1 expression. Although no PD-L1 staining was observed in primary osteosarcoma tumors, populations of PD-L1 positive cells were observed in a majority of metastatic osteosarcomas (Figure 1A). Five

additional metastatic samples, without matched primary tumors, were obtained and stained for PD-L1. All of these additional metastatic tumors were positive for PD-L1. Of the sixteen patients examined for PD-L1 expression, twelve had positive expression of PD-L1 within the metastatic tumor (approximately 75%). Overall, PD-L1 staining varied considerably between patients, as well as within each tumor, with some metastatic lesions expressing high levels of PD-L1 and others expressing lower levels. Our data confirms results from a recent paper by Shen et al., where they saw a high amount of PD-L1 positive stain in metastatic osteosarcoma [36]. Additionally, we found a significant correlation between PD-L1 positive staining and CD8 tumor infiltrating lymphocytes in metastatic osteosarcoma, $r^2 = 0.73$, suggesting that these tumors are immunogenic, but able to tolerize infiltrating T cells within the tumor microenvironment via PD-L1 interactions.

4.2. CTL in human metastatic osteosarcoma express PD-1

We next asked if PD-1 was being expressed on tumor infiltrating lymphocytes during metastatic osteosarcoma progression. Matched primary and metastatic osteosarcoma sections from the eleven patients were stained for PD-1 as well as the five additional metastatic samples without matched primary tumor tissue. As expected, PD-1 expression was observed on tumor-infiltrating CD8 T cells in the majority of human metastatic osteosarcoma tissue samples (Figure 1B), but not in primary tumors from the same patients. PD-1+ cells appeared to be clustered throughout lesions, a pattern that has been observed during PD-1 staining in other tumors [37, 38]. Thirteen patients had populations of PD-1+ lymphocytes within the metastases, with a high correlation between PD-L1 and

PD-1 expression: only one tumor that was PD-L1 positive had no PD-1 staining. Interestingly, two of the PD-L1 negative tumors exhibited positive staining for PD-1 suggesting that PD-L1 may be expressed within these tumors but was not observed in the samples analyzed.

4.3. CTL slow progression of metastatic osteosarcoma

In order to evaluate if CTL can slow progression of metastatic osteosarcoma, with the potential for downstream blockade of PD-L1 to reinvigorate CTL and promote metastatic osteosarcoma tumor rejection, we depleted mice of CD8 T cells prior to K7M2 tumor implantation. This cell line originated from a spontaneously occurring murine osteosarcoma and following implantation in mice causes pulmonary metastases. CD8 T cell depletion was confirmed to be >90% by flow cytometry of peripheral blood mononuclear cells (data not shown). Mice that had been depleted of CD8 T cells prior to K7M2 implantation had significantly decreased survival rates compared to intact mice given the same number of tumor cells (Figure 2). CD8 depleted mice had a median survival time of 13 days (± 7.493) post K7M2 injection compared to a median survival time of 23 days (± 3.951) post inoculation for intact control mice.

4.4. Expression of PD-L1 on K7M2 tumor cells

In order to evaluate the efficacy of PD-L1 blockade on slowing the progression of metastatic osteosarcoma, we again utilized the K7M2 ectopic metastatic osteosarcoma mouse model. K7M2 cells showed significant expression of PD-L1 compared to isotype control staining (Figure 3A); the mean fluorescence intensity (MFI) for isotype antibody

staining was 1848 (critical value = 87.2) whereas the MFI for PD-L1 antibody staining was 4073 (critical value = 98.7). A chi-squared test comparing the two peaks gave a $t(x)=53.1$, with 1 degree of freedom, resulting in an approximate p value of 0.01. We next determined if expression of PD-L1 on K7M2 cells continued after implantation and development of metastatic osteosarcoma *in vivo*. Consistent with PD-L1 staining patterns in humans, a subset (~30%) of K7M2 cells isolated from lung metastases exhibited further increased PD-L1 expression compared to K7M2 cells pre-implantation (Figure 3A). Thus, K7M2 metastatic osteosarcoma cells express PD-L1, with higher levels on some cells after implantation *in vivo*. We hypothesize that this is possibly due to immune editing in which tumor cells that express higher levels of PD-L1, and that are able to tolerize infiltrating CTL, are able to preferentially survive.

4.5. CTL infiltrating osteosarcoma metastases express PD-1

Because K7M2 metastatic osteosarcoma cells are PD-L1 positive, and CTL responses are able to initially slow K7M2 progression, we asked whether CTL that infiltrated K7M2 tumors become PD-1 positive. Approximately 45% of lymphocytes infiltrating K7M2 lung metastases at the time of euthanasia were CD8 T cells. Of these, the majority (>85%) of infiltrating CD8 T cells were also positive for PD-1 expression. This was in contrast to CD8 T cell populations in the spleen from the same mice that were uniformly low for PD-1 expression (Figure 3B, C, and D). In addition, we were unable to recover CD8 T cells from perfused lungs of healthy mice, suggesting that T cells isolated from metastatic osteosarcomas are responding to the tumor, rather than non-specific T cells circulating through the lung tissue (data not shown).

To determine if expression of PD-1 on tumor infiltrating CTL in the K7M2 metastatic osteosarcoma mouse model is similar to that observed in human metastatic osteosarcoma, we analyzed PD-1 expression on T cells in lung tissue sections from tumor bearing mice by immunofluorescence. We observed some PD-1 positive infiltrating lymphocytes in early K7M2 metastatic osteosarcoma tumors, with higher amounts of staining observed later during disease progression (Figure 3B). No PD-1 expression and few to no tumor infiltrating lymphocytes were observed in healthy lung tissues. Thus, we propose that a majority of osteosarcoma-infiltrating CD8+ T cells express PD-1, which binds to PD-L1 on tumors, resulting in potent inhibition of T cell function, allowing for tumor progression. This may be due to direct suppression of tumor-reactive cells, or indirectly through suppression of infiltrating cells that may have alternative specificities. Taken together these results support the idea that the K7M2 implantable osteosarcoma metastatic model is similar to human metastatic osteosarcoma in terms of PD-1/PD-L1 expression and CTL inhibition and therefore, evaluating efficacy of PD-L1 antibody blockade within the K7M2 metastatic osteosarcoma model should be relevant to expected effects during treatment of human metastatic osteosarcoma.

4.6. Osteosarcoma specific CTL are deficient in cytokine production and cytotoxic function:

To determine if PD-1 positive CTL infiltrating K7M2 metastatic osteosarcomas were functionally impaired in their ability to reject tumors, CTL were isolated from lungs of tumor bearing mice and evaluated for their ability to produce IFNγ, TNF, and IL-2, key cytokines produced by T cells to promote tumor rejection and that are lost during T cell

exhaustion. Specifically, tumor-infiltrating lymphocytes were incubated with or without K7M2 cells and cytokine production evaluated by intracellular cytokine staining. CD8 T cells isolated from tumors and incubated without additional K7M2 cells were positive for IFNγ, TNF, and IL-2 production, suggesting that CTL are being triggered by tumor antigens *in vivo*. However, minimal IFNγ, TNF, or IL-2 (Figure 4A, 4B, 4C) production was observed in CTL isolated from tumors after incubation with K7M2 cells *in vitro*. Thus, the presence of K7M2 tumor cells inhibited cytokine production by CTL.

To determine if CTL suppression of cytokines was due to PD-L1 ligation to PD-1 on CTL, we used a lentiviral vector to knock-down PD-L1 on the K7M2 cells, and used these cell to stimulate CTL isolated from tumor tissue in implanted mice. As expected, the decreased expression of PD-L1 by K7M2 cells reinvigorated CTL cytokine production of IFNγ, TNF, and IL-2 by tumor infiltrating T cells. Similarly, to determine whether the inhibition of cytokine production in the presence of K7M2 tumor cells was due to T cell tolerance to any antigen stimulation versus specific inhibition by K7M2 cells, we pulsed 4T1 mouse mammary tumor cells, which do not constitutively express PD-L1[39], with K7M2 cell lysate and used these to stimulate lymphocytes isolated from metastases in tumor-bearing mice. Again, we observed higher levels of IFNγ, TNF, and IL-2 production by CD8 T cells in response to 4T1 cells pulsed with K7M2 lysate compared to T cells incubated with non-pulsed 4T1 (Fig 4A, 4B, 4C).

To determine if the exhaustion of tumor infiltrating T cells included other effector functions, we next performed a cytotoxicity assay to determine if tumor-infiltrating T cells exhibited impaired killing of K7M2 cells. Tumor-reactive T cells were unable to specifically lyse K7M2 cells but were able to kill K7M2 cells that had been transduced

with lentiviral vectors to knock-down PD-L1 expression, suggesting that this pathway limits the function of these cells *in vivo*. Moreover, this data also suggests that TILs can be reinvigorated by tumor antigens in the absence of PD-L1 expression (p-value < 0.0001). To determine if this suppression of T cell function was specific for the K7M2 cells we also tested the ability of these cells to recognize and lyse the unrelated PD-L1-negative 4T1 mouse mammary tumor cells pulsed with K7M2 tumor lysate (Fig 4D). We observed specific lysis of 4T1 tumor cells pulsed with K7M2 tumor antigens, suggesting that T cell tolerance in this setting is due to specific inhibition by K7M2 cells as opposed to lack of antigen stimulation (p-value of <0.0001). Thus, CTL are impaired in the presence of K7M2 metastatic osteosarcoma tumor cells, likely due to PD-1 ligation by PD-L1. Moreover, removal of this inhibitory pathway suggests that CTL infiltrating metastatic osteosarcomas are have the capacity to regain function during blockade of this pathway.

4.7. Blockade of PD-L1 significantly enhances survival and improves disease state.

Based on the *in vitro* restoration of function in T cells from K7M2 metastatic osteosarcomas in the absence of PD-L1, we next determined if monoclonal antibody (mAb) blockade of PD-1/PD-L1 interactions could impact disease progression *in vivo*. Following implantation of K7M2 tumor cells, mice were administered PD-L1 mAb over a span of 15 days. We observed significantly increased survival of mice treated with PD-L1 mAb compared to those treated with isotype control antibody (Figure 5A). PD-L1 blockade doubled the median survival time of treated mice to 48 days post K7M2 inoculation compared to a median survival time of control treated mice of 24 days.

Moreover, even though PD-L1 mAb treated mice eventually succumbed to disease, the number of lung metastases was far less compared to control mice (Figure 5B); the few metastases in PD-L1 mAb treated mice eventually grew to a much larger size, causing disease at a later time.

4.8. PD-L1 mAb blockade improves the function of metastatic osteosarcoma infiltrating CTL *in vivo.*

We next asked whether the improved control of K7M2 metastases in PD-L1 mAb treated mice was due to improved function of tumor-infiltrating CTL. CTL from K7M2 tumors of mice treated with PD-L1 mAb or control injections as above, were isolated. CD8 T cells isolated from PD-L1 mAb treated mice were able to produce TNF and IL-2 even in the presence of K7M2 osteosarcoma cells *ex vivo,* while CTL from control-treated mice again had minimal cytokine production in the presence of K7M2 cells (Figure 6B and C). Interestingly, no difference in IFNγ production was observed between PD-L1 mAb treated and controls incubated in the presence of K7M2 cells, consistent with previous reports on the differential role this receptor may play in regulating these cytokines (Figure 6A)[40].

Conclusion:

Despite aggressive chemotherapy and surgery, the prognosis for patients with metastatic osteosarcoma remains dismal, in part due to the relatively resistant nature of osteosarcoma to conventional chemotherapy or radiation treatments. Osteosarcoma also typically lacks hallmark genomic alterations that might be targeted with specific

molecular therapeutics[41]. Immunotherapy, via blockade of inhibitory receptor pathways to reinvigorate endogenous T cell responses, provides a novel approach for therapy of metastatic osteosarcoma, whereby the power of the host immune system can be harnessed to improve tumor control. We have shown that both human and murine metastatic osteosarcomas express the PD-L1 inhibitory receptor, and provide evidence suggesting that tumor infiltrating CTL are functionally impaired due to ligation of PD-1 by PD-L1. Moreover, blockade of PD-L1 improves CTL function *in vivo* and results in significant, albeit incomplete tumor control. Thus, PD-L1 mAb blockade is an attractive candidate for immunotherapy in humans to enhance T cell mediated rejection of metastatic osteosarcoma and potentially improve patient prognoses.

Although T cell exhaustion was initially described in a chronic viral infection setting, with loss of T cell proliferation, cytotoxicity, and cytokine production, the severity of CTL tolerance in tumor settings is often much less pronounced, likely depending on the overall levels of antigen stimulation as well as the number and type of inhibitory receptors expressed. Thus, CTL exhaustion appears to be a progressive loss of effector functions, in which proliferative responses, cytotoxic responses, and/or cytokine production are lost in a step-wise manner depending on the milieu of inhibitory signals (REFERENCE WHERRY). Other tumor models have also shown incomplete T cells exhaustion during tumor progression, and T cell effector function can be reinvigorated in these settings through selective blockade of inhibitory T cell pathways. For example, abrogation of SHP-1, a phosphatase that inactivates downstream proteins in the PD-1 pathway[42], increases proliferation and cytokine production of T cells responding to FBL leukemia [43]. We have shown that blockade of PD-L1 on metastatic osteosarcoma

can reinvigorate T cell cytokine production, further supporting the idea that this is a key pathway in regulating CTL function during tumor progression.

An interesting observation in these studies is that PD-L1 blockade in the K7M2 metastatic osteosarcoma model restores CTL production of TNF and IL-2 in the presence of tumor cells but did not restore IFNy production. Other inhibitory pathways, including those modulated by TIM3, LAG3, CTLA-4, and IDO, can selectively dampen T cell cytokine production during tumor progression[44-49]. Thus, it is likely that these other pathways continue to regulate CTL function even during PD-L1 blockade and combinational therapies may be necessary to completely rescue T cell function, and subsequently to provide complete control of metastatic osteosarcoma. Conversely, PD-1 blockade coupled with immune stimulation, such as by addition of agonistic antibodies against OX40 or 4-1BB, may also improve CTL function within metastatic osteosarcoma[50, 51]. Combining PD-L1 blockade with CTLA-4 blockade, and addition of exogenous IL-2, has been shown to enhance T cell cytokine production and result in overall increases in survival in several tumor settings[48, 52-54]. Moreover, combinational PD-L1 and CTLA-4 blockade has shown clinical efficacy in the treatment of malignant melanomas, with 53% of patients exhibiting greater than 80% reduction in tumor size [55]. Given the previous results showing the importance of CTLA-4 and TIM3 in metastatic osteosarcoma, such combinational blockade studies should provide further enhancement of immunotherapy efficacy.

Despite the reinvigoration of cytokine production in osteosarcoma infiltrating CTL, mice treated with PD-L1 blockade eventually succumb to disease. In the K7M2 metastatic osteosarcoma model, this manifests as far fewer but much larger tumors. There

are two likely explanations for delayed disease due to metastatic osteosarcoma in PD-L1 antibody treated mice: 1) either tumor immune editing is occurring, selecting for a more aggressive metastatic tumor that is able to overcome T cell function, or 2) incomplete control of metastatic osteosarcoma is occurring early on during treatment, with T cell exhaustion setting in later. Our data favors the former explanation as PD-L1 mAb treated mice have increased CTL function, but eventually die from tumor burden due to incomplete T cell control.

Recently, a phase I/II clinical trial evaluating efficacy of PD-1 blockade on other advanced cancer types that are non-responsive to traditional treatments, including non-small-cell lung cancer, melanoma, renal-cell cancer, ovarian cancer, colorectal cancer, pancreatic cancer, gastric cancer, and breast cancer, demonstrated the efficacy of this approach for improving patient prognosis as 17% of patients experienced tumor regression and 41% had prolonged stabilization of disease[31]. This treatment was also safe, as minimal side effects of PD-L1 blockade were observed compared to conventional radiation and chemotherapy approaches. The Children's Oncology Group is currently designing a similar Phase I trial of anti-PD-1 inhibitor for children with relapsed and refractory solid tumors. Based on our results, we have provided the necessary pre-clinical data to support the use of PD-L1 mAb blockade for treatment of metastatic osteosarcoma in pediatric patients.

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Figure legends:

Table 1: Human osteosarcoma patient information. Human tissue used for evaluation of PD-L1 and PD-1 expression. R=right, L=left, +=positive low level of expression (less than 10 cells/field of view), ++=positive high level of expression (great than 10 cells/field of view), -=negative expression.

Figure 1: PD-L1+ and PD-1+ expression in human metastatic osteosarcoma tissue. A. Paraffin embedded human primary and metastatic osteosarcoma tissue was stained with 1:200 PD-L1 antibody (Abcam ab174838), detected using a Alexa Fluor 488 tagged secondary, B. or 1:200 PD:1 antibody (Abcam ab52587), detected using Texas Red tagged secondary, compared to isotype staining. Leica TCS SP5 confocal microscope was used to detect and produce images. Images are maximum intensity projections of several 0.8um confocal sections. Counterstained with DAPI 1:2000. Scale bars, 50um. Twelve out of sixteen pediatric patients (approximately 64%)with metastatic disease were PD-L1+. Thirteen out of sixteen pediatric patients (approximately 73%) were PD-1+. These sections are four representative patient samples. C. Multiple fields of view from patients stained with anti-CD8 and anti-PDL1 show a strong correlation between PD-L1 positive expression and number of CD8 infiltrating T cells.

Figure 2: CTL slow progression of metastatic osteosarcoma. Balb/cJ mice were injected with 10^6 K7M2 osteosarcoma cells post CD8 depletion. Survival was significantly decreased in mice depleted of CD8 cells (dashed), compared to wild-

type mice (solid). Log-rank (Mantel-Cox) test showed a significant p value <0.0001. CD8 depleted group had an n=10, while the wild-type K7M2 injected group had an n=55.

Figure 3: Expression of PD-L1 and PD-1, on K7M2 cells and tumor infiltrating lymphocytes, in a mouse model of metastatic osteosarcoma. A. PD-L1 is expressed on K7M2 osteosarcoma cell line, and upregulated after implantation**.** K7M2 cells were stained with anti-PD-L1 antibody (black) or isotype control staining (IgG2a λ, line). MFI for each peak is indicated on graph, showing significant differences between isotype control and PD-L1. Additionally, 10^6 K7M2 cells were injected into a Balb/cJ mouse, and stained for PD-L1 (black). After implantation, a subpopulation of cells (approximately 30%) expressed high levels of PD-L1. B. Immunofluorescent detection of PD-1. Mouse metastatic osteosarcoma tissue was stained with PD-1 antibody, and compared to isotype staining. Leica TCS SP5 confocal microscope was used to detect and produce images. Images are maximum intensity projections of several 1um confocal sections. Counterstained with DAPI 1:2000. Scale bars, 50um. C. 10^6 K7M2 cells were injected into Balb/cJ mice. Lymphocytes were isolated and stained with anti-PD-1 and anti-CD8 antibodies and compared to PD-1 expression on lymphocytes isolated from spleen. Flow data was analyzed using FlowJo8.8. D. Multiple mice showed the similar levels of PD-1+ CD8+ TILs. Unpaired T test gave a P value significance of <0.0001 comparing TIL CD8 (black) to CD8 isolated from spleen (open).

Figure 4: Osteosarcoma specific CTL are deficient in cytokine production. 10^6 Cells were stained for extracellular markers, and then fixed and permeabilized, and stained for IFNy, TNF, and IL-2 production. TIL CD8 were incubated with K7M2, 4T1 alone, 4T1 cells pulsed with K7M2 tumor lysate, or K7M2 transduced to be PD-L1 negative for 4 hours. Flow data were analyzed using FlowJo8.8, gating on the PD-1+ CD8+ population. White bars indicate cytokine production to no additional antigen (TILs incubated alone or with 4T1 not pulsed), while black bars indicate cytokine production to osteosarcoma cells (with or without PD-L1 expression) or osteosarcoma antigens. In each case, we are measuring the percentage of PD1+ CD8+ T cells that are expressing each of the three cytokines. A. Multiple T test comparing IFNy production by TIL CD8 alone versus co-cultured with K7M2 cells gave a P value= 0.0046. T test comparing IFNy production by TIL CD8 co-cultured with non-pulsed 4T1 versus pulsed 4T1 alone gave a P value= 0.021. IFNy production by TIL CD8 cultured with K7M2 cells compared to 4T1 pulsed with K7M2 lysate gave a P-value= 0.0437. Finally, IFNy production by TIL CD8 cultured with K7M2 cells compared to K7M2 that are PD-L1 negative gave a P-value= 0.001. B. Multiple T test comparing TNF production by TIL CD8 alone versus co-cultured with K7M2 cells gave a P value= 0.009. T test comparing TNF production by TIL CD8 co-cultured with non-pulsed 4T1 versus pulsed 4T1 gave a P value= 0.05. TNF production by TIL CD8 cultured with K7M2 cells compared to 4T1 pulsed with K7M2 lysate gave a P-value= 0.02. Finally, TNF production by TIL CD8 cultured with

K7M2 cells compared to K7M2 with negative PD-L1 expression gave a P-value= 0.002. C. Multiple T test comparing IL-2 production by TIL CD8 alone versus co-cultured with K7M2 cells gave a P value= 0.009. IL-2 production by TIL CD8 cultured with K7M2 cells compared to 4T1 pulsed with K7M2 lysate gave a P-value= 0.025. Finally, IL-2 production by TIL CD8 cultured with K7M2 cells compared to K7M2 with negative PD-L1 gave a P-value= 0.0107. Open bars signify TILs cultured alone, black bars signify TILs + antigen. D. LDH ELISA confirming lack of cytotoxicity via tumor-reactive T cells when cocultured with K7M2 tumor cells. This is compared to a control of 4T1 antigen presenting cells (APC) that are PD-L1 negative, either non-pulsed or pulsed with K7M2 tumor lysate, or K7M2 cells transduced to be PD-L1 negative. T test comparing effector responses to K7M2 versus antigen presenting cells pulsed with K7M2 tumor lysate was significantly different, $p<0.0001$. T test comparing effector responses to K7M2 versus K7M2 negative for PD-L1 was significantly different, $p<0.0001$.

Figure 5: Blockade of PD-L1 enhances survival and improves disease state. A. Balb/cJ mice were injected with K7M2. Mice were than given therapeutic anti-PD-L1 antibody injections (10F.9G2) i.p. over several days, and survival was compared to no treatment group. Survival was significantly increased in mice treated with anti-PD-L1 antibody injections (dashed), compared to wild-type mice (solid). Log-rank (Mantel-Cox) test showed a significant p value= 0.0005. PD-L1 mAb treatment group had an n=10, and the wild-type K7M2 injected group had an n=10. B. Implantable K7M2 mice treated with PD-L1 monoclonal antibody had a reduction in number of

pulmonary metastatic lesions**.** Mice treated with PD-L1 antibody had a significant decrease in number of lesions, $p<0.0001$. When mice finally succumbed to tumor, metastatic lesions were able to get much larger in size compared to control group, $p<0.0001$. Area of lesion, width and length, were measured in millimeters. Volume was calculated using equation $(width)^2 * (length/2)$.

Figure 6: PD-L1 mAb blockade improves the function of metastatic osteosarcoma infiltrating CTL *in vivo*. K7M2 cells were injected into Balb/cJ mice. Treatment group were started on 200ug PD-L1 monoclonal antibody (clone 10F.92G) repeated every three days for 1000ug total. TILs were collected at time of death, and incubated with (black) or without (white) K7M2 cell stimulation for 5 hours, with GolgiStop. After incubation, cells were stained for extracellular proteins, then fixed and permeabilized, and stained for TNF, IL-2, and IFNy production, and compared to TNF, IL-2, and IFNy production from non-treated K7M2 injected mice. Flow data were analyzed using FlowJo8.8, gating on the PD-1+CD8+ population. A. Multiple T test comparing TNF production of TIL CD8 cultured alone or co-cultured with K7M2, without PD-L1 treatment, gave a P value= 0.01. Multiple T test comparing TNF production of TIL CD8 cultured alone or co-cultured with K7M2, with PD-L1 treatment, gave a P value= 0.032. Finally, multiple T test comparing TNF production of TIL CD8 cultured with K7M2 in control versus treated mice gave a P value= 0.0346. B. Multiple T test comparing IL-2 production of TIL CD8 cultured alone or co-cultured with K7M2, without PD-L1 treatment, gave a P value= 0.009. Multiple T test comparing IL-2 production of TIL CD8 cultured alone or co-cultured

with K7M2, with PD-L1 treatment, gave a P value= 0.031. Finally, multiple T test comparing IL-2 production of TIL CD8 cultured with K7M2 in control versus treated mice gave a P value= 0.0059. Number of mice in osteosarcoma group non-treated, n=10. Number of mice in osteosarcoma group treated, n=10. Open bars indicate TIL cultured alone. Black bars indicate TILs cultured with antigen. C. No significant differences where seen when multiple T test compared IFNy production of TIL CD8 cultured with K7M2 in control versus treated mice, P value =0.1204.

Table:

Case #	Age at primary Biopsy (year)	Age at metastasis Biopsy (year)	Primary Tumor site	Metastasis site	Biopsy decalcification	Metastasis decalcification	PD-L1 metastatic expression	PD-1+ TIL metastatic expression
1	7	9	R radius	L iliac bone & soft tissue	No	No	+	+
2	8	9	L distal femur	Mediatinum, thymus	Yes	No	+	+
3	14	18	R distal femur	Lung	No	No	+	-
4	12	14	R distal femur	Lung	Yes	No	++	++
5	14	15	L distal femur	Lung	No	Yes	-	-
6	13	14	R femur	Lung	No	No	-	+
7	12	15	L proximal tibia	Lung	Yes	No	+	++
8	10	10	L humerus	Lung	Yes	Yes	-	-
9	15	21	R femur	Lung	Yes	No	-	+
10	10	12	R shoulder	Lung	No	No	+	++
11	9	10	R distal femur	Lung	Yes	No	++	++
12	15	15	N/A	Lung	Yes	Yes	+	+
13	15	21	N/A	Lung	Unknown	No	+	+
14	8	9	N/A	Lung	Unknown	Yes	+	+
15	16	16	N/A	Lung	No	No	++	++
16	16	18	N/A	Lung	No	No	++	+